FAMILY GROWTH RESPONSE TO FISHMEAL AND PLANT-BASED DIETS SHOWS GENOTYPE X DIET INTERACTION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Lindsey R. Pierce

(Abstract)

The ability of rainbow trout to efficiently utilize plant-based diets for growth and the genetic variation for that trait have not been thoroughly examined. In this study, growth of a pedigreed population from the commercial Kamloop strain was assessed while feeding plant-based or traditional fishmeal-based diets. Both fish oil (5.00%) and soybean oil (8.43%) were included in the plant-based diet, and only fish oil was used in the fishmeal diet (10.10%). Ninety-five full-sib families nested within 47 half-sib families were reared in a common environment. Parentage assignment was performed on approximately 1,000 fish fed each diet using eight microsatellite markers chosen for non-duplication, a minimum of five alleles with no known null alleles, at least 50% heterozygosity, and unambiguous scoring. Progeny were assigned to parental pairs using two allocation programs, PAPA and FAP, to increase accuracy and to test assignment efficiency. The fish fed the fish meal/oil diet were approximately 8% larger than the fish fed the plant-based diet ($P < 0.05$). A significant genotype x diet effect accounted for 5% of the random variation. The genetic correlation for growth on the two diets was 73%, with a heritability of 30% across the diets. With this, I conclude that substantial genetic variation for utilizing plant-based diets containing soybean meal and oil exists in this widely used commercial rainbow trout strain. The genetic variation can be explored to detect and select for genes involved in improved utilization of plant-based diets

containing soybean meal and oil if growth on plant-based meals becomes a long-term breeding goal in rainbow trout production.

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Chapter I

Introduction

Need for plant-based aquaculture diets

As the demand for fisheries products rises, it is no longer feasible to rely solely upon harvest from the wild. The dramatic development of fish farming has allowed economic production of a healthy, high-quality product for the consumer, and has provided a reliable source of fisheries products. The Food and Agriculture Organization of the United Nations (2007) reported that aquaculture is one of the fastest-expanding agricultural industries in the world, with growth rates in excess of 30 percent per year, more than three times that for growth in terrestrial farm animal meat production. Additionally, UNFAO (2007) stated that by 2004, aquaculture had provided nearly 50 percent - or 59.4 million tons - of all world fisheries production.

Of the seven major costs for fish farming – feed, labor, animals, energy, processing, marketing, and distribution – the price of feed can account for as much as 55% of variable production costs (Helfrich 1997). With the continuing growth of the aquaculture industry, large amounts of oceanic forage fish are being exploited for fish meal or fish oil for commercial feeds, thereby causing negative ecological and environmental impacts. A total of one-third of the fish caught each year from oceanic waters are used for feed production (Goldburg et al. 2002).

Most fish feeds incorporate fishmeal or fish oil to meet protein and essential fatty acids requirements. In part because of rising demand from aquaculture, the production price of fishmeal and oil have increased to almost three times the price of soybean

meal/oil diets (Miles and Chapman 2006; FAO Globefish 2008). New (2002) predicted that the global demand for fishmeal in aquafeeds will exceed total available supplies around the year 2020, and that fish oil supplies will be depleted by 2010. Hence, there are increasing efforts to incorporate alternate, plant-derived ingredients in aquaculture feeds (Gomes et al. 1995; Hardy 1996; Sugiura et al. 1999; Carter and Hauler 2000; Kissil et al. 2000, Barrows et al. 2007).

Quinton et al. (2007), working with European whitefish (*Coregonus lavaretus L.*), studied selection response to quantify the impact of genotype x environment interaction when fishmeal or soybean meal-based diets were administered through grow-out. Tests of trait expression for diet-specific phenotypic and genetic variation and between-diet genetic correlations were examined to determine whether family response to diet could provide the basis for improved breeding strategies utilizing family selection should family-based selection prove effective. In agreement with Palti et al. (2006), Quinton et al. (2007) found that the between-diet differences in growth proved insignificant in a related feeding trial. Additionally, Quinton et al. (2007) discovered that the genetic correlations between diets indicated little re-ranking of families, implying that current selection on fishmeal diets will lead to strong performance on SBM diets.

Complexities posed by developing plant-based diets

For aquaculture production, a complete diet is required since the animal cannot obtain essential nutrients for growth and reproduction from its food web. In the wild, consumption of algae and zooplankton would allow a fish to maintain sustainable levels of n-3 fatty acids and highly-unsaturated fatty acids (Hardy and Shepherd 2006);

however, farmed fish must be fed a complete diet of protein, carbohydrates, fats, vitamins, and minerals for optimum growth and health.

Rainbow trout is an important aquaculture species in the United States. With ninety-five percent of rainbow trout (*Oncorhynchus mykiss*) consumed in the U.S. being farm-raised, many studies have been done to increase feed efficiency and production. Rainbow trout (*Oncorhynchus mykiss*) is a carnivorous species, meaning that it requires high levels of protein and fat and that it digests carbohydrates poorly. In order to obtain maximum growth rate, maintain water quality, support effective osmoregulation, and minimize disease, use of appropriate amounts of quality feed plays a major role. For *Oncorhynchus mykiss*, 1600-1650 metabolizable kilocalories (kcal) of energy are required per pound of weight gain (Klontz 1991). Watanabe et al. (1979) and Cho (1982) showed that 35 to 36 percent protein and 15 to 16 percent lipid were optimum for weight gain in rainbow trout. To account for these requirements, most commercial feeds contain fishmeal, an expensive ingredient.

Since rainbow trout is a carnivorous species, and generally consumes greater than twenty percent fish products, feed formulations that incorporate other, less expensive protein sources are becoming highly sought. However, replacement of fish protein and oil sources in the diet by plant-derived protein and oil poses technological and biological challenges. Many plant byproducts contain lower protein levels and lack essential amino acids that fish meal provides (Adelizi et al. 1998). In addition, plant meals can contain anti-nutritional factors such as trypsin inhibitors, non-digestible carbohydrates, lectins, saponins, phytates, and possibly- allergenic storage proteins (Salunkhe et al. 1992). Decreased palatability, which in turn could lead to decreased consumption, is also a

concern when fishmeal is replaced by plant-derived ingredients (Davis et al. 1995; Stickney et al. 1996).

Development of fish stocks that can utilize plant-based feeds effectively

The anticipated changes in feed formulations have raised concerns regarding the ability of fish selected for rapid growth on traditional, fishmeal-based diets to effectively utilize these alternate diets (Blanc 2002). Only limited research has aimed at quantifying the ability of cultured stocks of carnivorous species such as rainbow trout to utilize plant-derived diets, and to determine whether there is a genetic basis for ability to perform well on such diets. Palti et al. (2006) tested family growth response to fishmeal- and plantmeal-based diets in the widely-used "spring" rainbow trout strain to assess the magnitude of genotype x diet interaction. In this experiment, both diets produced similar growth results.

While other studies have shown little to no family response, other research should be conducted with alternate sampling strategies, plant-based diet formulations, and salmonid strains. Should a genotype x diet interaction be observed in a family study under new sampling techniques, geneticists then can use selective breeding to maximize feed efficiency and profit for the producer.

Utility of common-garden experimental design

The ability to distinguish family groups could allow use of a "common garden" experimental design. Due to the small size of rainbow trout juveniles, physical marking is not practical. The ability to identify parent-offspring relationships, by analysis of multi-

locus genotype data has revolutionized the study of both natural populations and managed stocks (Taggart 2006). By allocating genotypes, parental assignment programs have allowed researchers to use mixed-family experimental designs by distinguishing membership among the different families used.

Project Goal and Objectives

Against this background, the goal of this study was to assess family growth response to fishmeal- and plant-based diets and evaluate whether a genotype x feed interaction exists. The objectives of my research were to:

1. Use microsatellite DNA marker-based pedigree assignment to accurately allocate progeny to sib-groups in a common garden experimental design, and to

2. Examine growth parameters using a family means approach to compare the performance of progeny from each full-sib and half-sib family fed a traditional fish-meal based diet or a plant-protein based diet.

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Chapter II

Microsatellite marker-based assignment of parentage to mixed families of rainbow trout

Introduction

Genetic improvements of aquaculture stocks have been reported with increasing frequency (Gjedrem 2000). Results from commercial rainbow trout (*Oncorhynchus mykiss*) breeding programs have shown gains of approximately 15% per generation from selection for body size (James Parsons, Troutlodge, Inc., unpublished data). Many aquaculture selection programs utilize a family-based mating design and benefit from the high fecundity of most aquatic species; external fertilization, which enables simultaneous multiple matings; and use of semen storage and cryopreservation for fertilizations. Full- and half-sib families possess appropriate genetic relationships for estimating breeding values and genetic correlations among traits of interest (Falconer and Mackay 1996). However, difficulty in marking small fishes often has necessitated the rearing of early developmental stages in individual family tanks, resulting in the need for many replicated tanks and causing shared-tank effects among family members reared together (Winkelman and Peterson 1994). Additionally, aquaculture performance when families are reared separately is not necessarily representative of performance in mixed-family tanks (Herbinger et al. 1999). The use of genetic markers for assigning parentage and for pedigree analysis in "common garden" aquaculture experiments has become reasonably common (O'Reilly et al. 1998; Fishback et al. 1999, 2002; Herbinger et al. 1999; Hara and Sekino 2003; Sekino et al. 2003; Vandeputte et al. 2004; Palti et al. 2006) and allows evaluation of genotype x environment effects without confounding common-environment

effects. However, the high cost of the molecular genetics techniques needed to support these analyses has limited the use of "genetic tagging" by commercial breeders. Recently, Johnson et al. 2007 developed a microsatellite multiplex system for rainbow trout to effectively reduce the cost of reagents and time associated with pedigree allocation and genetic tagging in common garden breeding designs.

Algorithms based on inclusion or exclusion of possible parents have been developed to assign parentage of individuals produced in the wild and in culture contexts (Jones and Ardren 2003). These algorithms may be applied by a growing number of computer software packages, among them PAPA (Duchesne et al. 2002) and FAP (Taggart 2006). Well-chosen combinations may have complementary strengths. In this study, I assigned parentage in a mixed group of rainbow trout using eight microsatellite markers using both PAPA and FAP.

Methods

Fish Stocks

The Kamloop strain of rainbow trout from Troutlodge, Inc. (Sumner, WA, USA), which has been selected for improved growth for three generations using a best linear unbiased predictor (BLUP)-supported breeding value assignment program, was the base population for this study.

Mating design and early rearing

One-hundred-and-sixty (160) females were mated to 80 males on (Table 2.1) on the basis of pedigree and breeding values using the following approach: Each male fertilized the eggs of two females, using the criterion that the parents of each cross could have no common grandparent. The parents were fin-clipped for subsequent DNA isolation and analysis. All the fertilizations occurred between August 9-15, 2005. Upon fertilization, a common egg displacement procedure was applied (Palti et al. 2006). The degree-day of incubation of all lots was matched, and they reached the "eyed" stage on the same day. Family numbers then were reduced to 95 full-sib families (approximately 9,500 eggs) within a 47 half-sib group (Figure 2.1). The same number of eggs per family then was pooled among all families, mixed well, and randomly split into two groups that were incubated separately in hatching jars through hatching until the initiation of feeding.

At the start of feeding, the swim-up fry were placed into six 6,000-L rearing tanks supplied with spring water (12ºC). Feeding was as described below. Bi-weekly sampling of weight continued through grow-out, and feeding rates were adjusted. Fish were transferred from rearing tanks to standard concrete grow-out raceways at an average weight of 25 g and grown out until harvest at approximately 600 grams.

Diet formulation and feeding regime

Two diets were formulated to be isonitrogenous and isolipidic (Chapter 3; Table 3.1), one a traditional fish-meal-based diet and the other a plant-based diet. The diets were manufactured at the Feed and Nutrition Laboratory of the U.S. Fish and Wildlife Service Fish Technology Center in Bozeman, MT for 0.5 to 3.0 mm pellets. Additional 4.5 mm pellets were produced by a commercial mill (Nelson and Sons, Murray, UT).

Krill meal (5%) was added to the plant-based diet to increase palatability. Astaxanthin, a flesh-coloring agent, was included in the 4.5 mm pellets.

Sampling for parentage analysis

Once harvest weight was reached, a random sample of 1,032 fish from each replicate (344 per raceway) was taken for measuring length and weight and for fin-clipping. The fin-clips obtained from these fish were stored in 100% ethanol until DNA extraction (Palti et al. 2002).

Markers and genotyping

Of the initial 2,068 fin clips collected, 1,996 were used for DNA extraction. The 72 were not extracted due to sample degradation or loss. After DNA extraction, purification, and quantification, the samples were diluted to 12.5ng/µl and used for polymerase chain reaction (PCR). Microsatellite multiplexes (Johnson et al. 2007) were used to decrease cost and laboratory time. However, two of the 12 markers were eliminated due to the presence of null alleles or genetic linkage with other markers in the multiplex. The 10 markers used were: *OMM5132*, *1008*, *5007*, *5047* and *5233* in multiplex 1 and *OMM5177*, *1051*, *1097*, *1088* and *1325* in multiplex 2. PCR conditions followed Johnson et al. (2007), with modified cycling times as follow. For multiplex 1: 95^{o}C for 10 min; 2 cycles of 94^{o}C for 1 min, 62^{o}C for 45 sec, 72^{o}C for 2 min; 29 cycles of 94^{o}C for 1 min, 58^{o}C for 45 sec, 72^{o}C for 2 min; 72^{o}C for 45 min; 4^{o}C for 1 hr, and 12^{o}C hold. For multiplex 2, 95^{o}C for 10 min; 29 cycles of 94^{o}C for 1 min, 58^{o}C for 45 sec, 72^{o}C for 2 min; 72^{o}C for 45 min; 4^{o}C for 1 hr; and 12^{o}C hold. Amplifications were

conducted on a Research DNA Engine thermal cycler (Model PTC 200, MJ Research, Waltham, MA, USA). PCR amplification products were verified on 3% agarose gels stained with ethidium bromide and then diluted according to intensity. Three µl of each PCR product were diluted in 20µl water, and 1 µl of the diluted product was mixed with 0.13µl Rox-labeled 400 bp size standard and12µl HiDye-formamide. After denaturing, an ABI 3730 DNA Genomic Analyzer was used for fragment separation and visualization. The output data were analyzed using GeneMapper 3.5 software (ABI, Foster City, CA, USA).

Parental analysis

All parent (Table 2.2) and progeny genotypes were used as input for parental determination using the programs PAPA 2.0 (Duchesne et al. 2002) and FAP 3.5 (Taggart 2006). The two programs were used in order to reduce error, increase accuracy, and assess efficiency of parentage assignment. Individual progeny that were allocated differently between programs were evaluated and assigned manually to the correct full-sib families.

I evaluated the frequency distribution of the random sampling per sire family and diet to determine whether survival was differentially affected by diet within families and whether the overall distribution of sire families was different from the expected mean of 40 offspring. The Fit Ordinal Logistic function of JMP 5.0 was used assess the deviation of the overall distribution from the expected mean.

Results

Parentage assignment

All microsatellite markers used in the multiplex system proved informative. However, marker *OMM5132* was difficult to genotype due to the presence of alleles separated by only one base-pair (Johnson et al. 2007). An unexpectedly high rate of genotyping errors was observed for marker *OMM5233,* reducing the success of pedigree assignment for the marker set. Data from these two markers were removed from parentage analysis. Data for the other eight markers then were used for parentage assignment.

A total of 1,996 multilocus genotypes (992 for fish fed the fishmeal diet and 1,004 for fish fed the plant diet) were analyzed for parental assignment using both PAPA and FAP software packages (Table 2.3 and 2.4). I manually eliminated 34 samples for which I could not obtain genotypes for at least seven of the eight markers. Both programs were unable to assign the same 113 individuals to any one set of parents. In addition, four other progeny which could not be assigned to parents by PAPA were assigned by FAP. Another 29 progeny were assigned to different parents by the respective programs. Data for these individuals were examined manually to determine their correct parental allocation. Eight progeny could not be assigned to a single parental pair (i.e., the assignments were ambiguous), and nine progeny could be assigned only to the sire. I divided the remaining 12 progeny equally between the two programs, with six assigned by PAPA and six by FAP. Overall, I was able to assign parentage of both sire and dam to 1,841 progeny (909 for the fish meal diet and 932 for the plant-based diet), which were assigned to 92 full-sib families nested within 46 sire families.

All families were represented in the sample sets for both diets. The number of progeny per diet was very similar within sire families ($P > 0.45$ using paired T-test; Figure 2.2), which implies that diet did not have a differential effect on survival within sire families, and that sampling was not biased. The overall distribution of individuals among sire families was significantly different from the mean expected number of 40 offspring per sire ($P < 0.0001$), which was caused by the expected variation due to random sampling and by differential survival between sire families.

Discussion

The use of "common garden" experimental approaches in aquaculture has proven to be more efficient than tagging small individuals (O'Reilly et al. 1998). The use of common garden designs allows researchers to minimize tank effects while focusing on family and genotype x environment. Although some may argue that molecular marker-based inference of parentage is less efficient than rearing families separately in replicated tanks, I was able to compare the performance of 93 families using just six rearing units. I was able to correctly assign 98.4% of progeny to parents, a high percentage as seen in many other parentage assignment studies (O'Reilly et al. 1998; Fishback et al. 1999, 2002; Herbinger et al. 1999; Hara and Sekino 2003; Sekino et al. 2003; Vandeputte et al. 2004; Palti et al. 2006).

The common garden approach allows family-based performance testing on commercial farms under production conditions, i.e., it allows practical genetic evaluation as part of routine production activities. By using parentage assignment in "common

garden" breeding designs, on-farm selective breeding programs can produce stocks exhibiting desired traits. Genetic selection strategies such as "walk-back" selection (Doyle and Herbinger 1994) could be implemented to produce superior individuals without worry of tagging/handling. The effectiveness of "walk-back" selection, an intense within-family selection strategy, has been estimated to exceed that of combined selection by one to three standard deviations in aquaculture populations (Tave 1995). Genetic improvements in marker-assisted family selection could help reduce stress in fish and increase accuracy in assignment when choosing individuals for advanced broodstock selection.

Parentage programs

I used two parentage assignment programs, PAPA 2.0 (Duchesne et al. 2002) and FAP 3.5 (Taggart 2006), in order to increase family assignment accuracy. PAPA, which was used as the base program of this study, allowed restrictions on which parents, progeny, crosses, markers, and percent error to include. This permitted the user to limit the possible outcomes and to receive only the output information desired. Using FAP, individual cross-matings restrictions are not allowed. Therefore, I used FAP to analyze the possible assignments from the database of all progeny to each individual parental cross separately. The two programs produced very similar results, agreeing in 98.4% of their parental-pair assignments. For the remaining 1.6%, FAP had an advantage over PAPA because it identified all possible parental pairs, while PAPA identified only one pair, even where there was ambiguity. Although FAP was not intended for this experimental design, it proved useful for verification of PAPA's parental allocations. My

results suggest that for future research, both PAPA and FAP should be used simultaneously to increase assignment frequency and to verify accuracy.

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Table 2.1 Original parental mating design.

Fam	♀	♂
1	91	48
	120	
2	114	40
	86	
3	126	37
	112	
4	128	39
	106	
5	102	52
	89	
6	122	46
	119	
7	96	43
	105	
8	104	38
	95	
9	131	49
	127	
10	117	57
	93	
11	103	44
	115	
12	109	60
	123	
13	90	59
	94	
14	88	53
	113	
15	111	51
	124	
16	101	47
	87	
17	97	56
	129	

Fam	♀	♂
18	107	61
	108	
19	92	50
	116	
20	121	54
	130	
21	85	45
	110	
22	98	42
	100	
23	99	55
	118	
	125	
24	162	63
	160	
25	145	82
	134	
26	161	65
	150	
27	173	76
	157	
28	149	66
	165	
29	135	73
	159	
30	176	75
	166	
31	143	67
	168	
32	137	77
	175	
33	174	68
	138	

Fam	♀	♂
34	132	62
	133	
35	153	70
	171	
36	136	64
	148	
37	177	72
	178	
38	156	88
	169	
39	142	69
	154	
40	151	84
	152	
41	140	86
	141	
42	147	79
	167	
43	146	74
	163	
44	158	81
	170	
45	139	71
	179	
46	155	83
	180	
47	144	87
	157	

Table 2.2 Sample of parental genotypes used for assignment to mixed-progeny groups.

	Genotypes at given microsatellite loci[1]								
Parents	*OMM1051*	*OMM1088*	*OMM1097*	*OMM1325*	*OMM5177*	*OMM1008*	*OMM5007*	*OMM5047*	*OMM5233*
F091	243/243	125/125	289/293	280/280	129/135	267/270	170/174	260/274	133/135
F120	243/243	113/113	301/301	280/280	126/135	267/270	170/174	260/274	117/133
*									
F114	243/279	113/117	245/293	280/288	120/141	267/276	174/180	266/278	117/133
F086	243/243	113/129	273/293	280/288	126/129	258/267	174/188	260/274	131/131
*									
F126	243/243	125/141	233/301	280/288	126/129	267/267	160/166	260/278	133/133
F112	243/243	117/125	289/301	288/288	129/129	267/267	160/188	260/266	125/135
*									
M_059	243/279	117/129	229/273	280/288	129/129	267/267	170/174	260/274	117/135
*									
M_053	219/279	129/145	249/301	280/288	129/129	258/267	174/174	266/274	117/135
*									
M_051	247/267	113/145	293/293	280/280	126/129	267/273	160/170	260/278	117/135
*									
M_047	243/279	113/129	225/293	280/288	120/141	267/267	162/174	260/274	117/125
*									
M_056	255/279	113/117	249/301	280/288	120/129	267/276	170/196	266/274	135/135

1. Genotypes are shown as sizes of microsatellite alleles in base pairs. Each parent transmits one allele of each gene to each of its progeny.

Table 2.3 Sample of parental assignments inferred using PAPA (Duchesne et al. 2002).

Offspring	Male	Female
PP_0658	M_037	F112
PP_0887	M_037	F112
PP_1019	M_037	F112
FM_0212	M_037	F112
FM_0869	M_037	F112
FM_0905	M_037	F112
FM_0322	M_037	F126
FM_0653	M_037	F126
FM_0866	M_037	F126
PP_0306	M_038	F095
PP_0705	M_038	F095
PP_0940	M_038	F095
FM_0268	M_038	F095
PP_0913	M_038	F104
FM_0213	M_038	F104
FM_0313	M_038	F104
FM_0641	M_038	F104

Table 2.4 Sample of parental assignments inferred using FAP (Taggart 2006).

Record I.D.	Composite Genotypes	Missing loci	# Mismatch alleles	Families sharing composite genotype
PP_0070	243267 113113 233293 288288 129141 267270 160174 260274	0	0	F_115xM_044
PP_0181	243267 113113 293293 280288 129135 267267 160174 260266	0	0	F_103xM_044
PP_0206	247267 113113 233321 288288 129141 267270 160196 260274	0	0	F_115xM_044
PP_0238	243267 113113 253293 280288 129141 267267 174174 266274	0	0	F_103xM_044
PP_0296	243243 113113 277293 288288 129141 267267 160196 260274	0	0	F_115xM_044
PP_0379	243243 113113 293321 280288 129135 267267 160174 260260	0	0	F_103xM_044
PP_0444	243267 113113 293293 280288 129129 267267 174174 266274	0	0	F_103xM_044
PP_0542	243267 113113 293321 280288 135141 267267 160174 260266	0	0	F_103xM_044
PP_0676	243267 113113 253321 280288 129129 267267 174174 260274	0	0	F_103xM_044
PP_0756	243267 113113 253293 280288 129135 267267 160174 260260	0	0	F_103xM_044
PP_0921	243267 113113 253293 280288 135141 267267 160174 260260	0	0	F_103xM_044
PP_0932	243267 113113 277321 288288 129129 267270 174174 274274	0	0	F_115xM_044
PP_0968	243243 113141 233321 288288 129141 267267 160196 260274	0	0	F_115xM_044
FM_0092	243247 113113 233293 288288 129129 267267 174174 274274	0	0	F_115xM_044
FM_0163	243247 113141 277293 288288 129129 267270 174196 274274	0	0	F_115xM_044
FM_0245	243267 113113 253321 280288 129135 267267 174174 260274	0	0	F_103xM_044

Figure 2.1 Schematic representation of the 95 x 47 nested mating design, growth trial and parentage determination analysis.

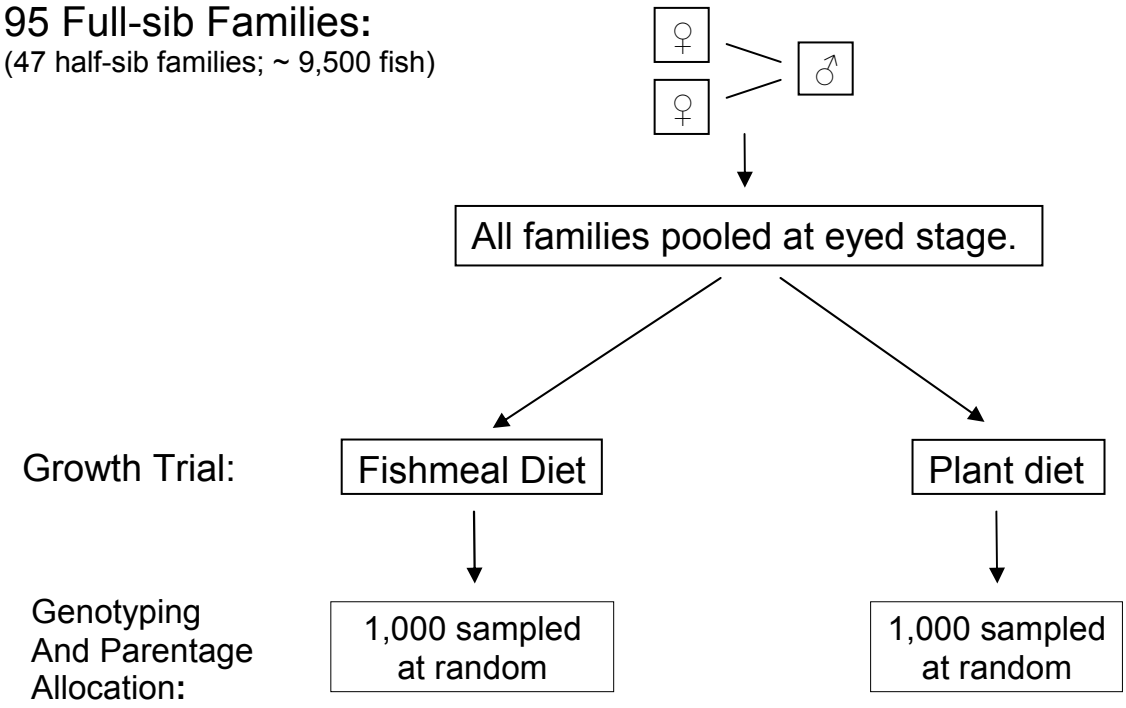
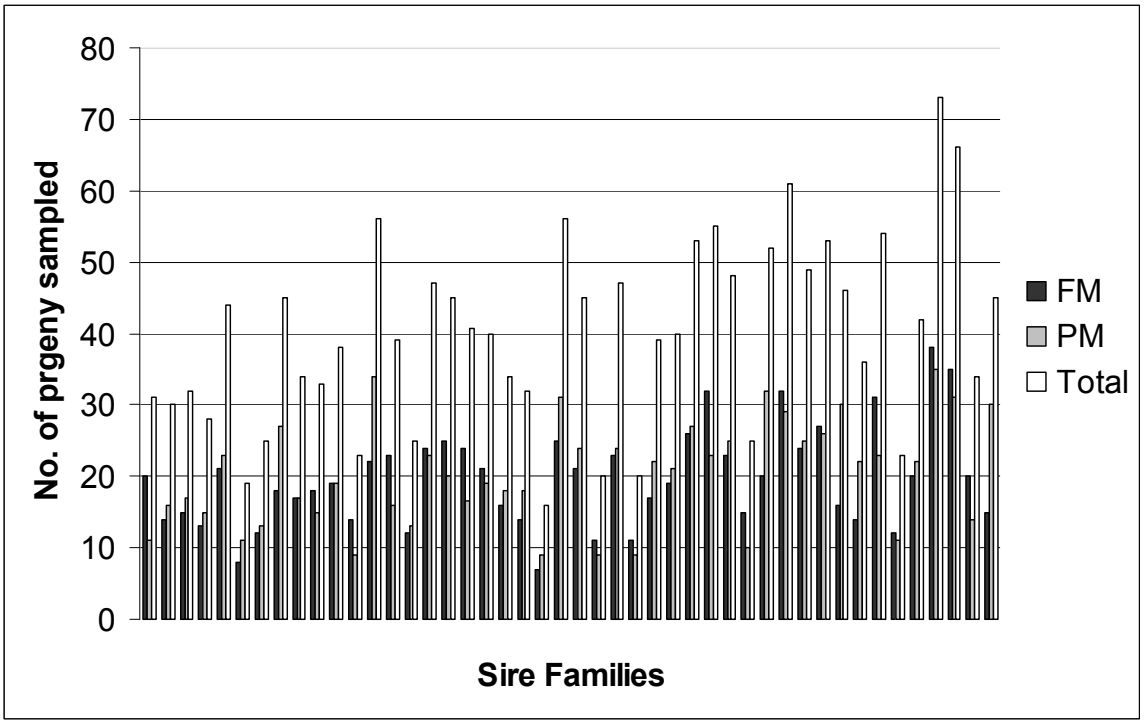


Figure 2.2 Distribution frequency of the number of progeny sampled at random from sire families (two half-sib families each) shown for each diet and a combined total for each sire. The distribution was significantly different from the expected mean of 40 progeny per sire ($P < 0.0001$), but the paired *T*-test comparison of the distribution by diet within sire families was not significant.



Chapter III

Evaluation of family growth response to fishmeal- and plant-based diets

Introduction

The aquaculture industry has been criticized for the large volumes of fishmeal and fish oil used in feeds, particularly those for salmonids. The harvest of forage fishes for fishmeal has been rather constant for several decades, and its limited availability imposes a major constraint on sustainable growth of global aquaculture production (Hardy 2006). Concern about overexploitation of forage fishes as feed ingredients (Goldburg et al. 2002) has prompted increased examination of alternate diet formulations for aquaculture.

As an aquaculture business expands, so does the need for high quality feeds. Of the seven major costs of trout farming – feed, labor, fish, energy, processing, marketing, and distribution – the price of feed can account for as much as 55% of variable production costs (Helfrich 1997). High protein and energy feeds are required by salmonids since they utilize carbohydrates poorly. In order to meet nutritional requirements, most commercial feeds contain animal protein (mostly fishmeal), which is an expensive ingredient. Fishmeal-based diets are typically more expensive then plant protein-based diets. The rising cost of high-quality fishmeal (65% protein), now up to three times the price of soybean meal (Miles and Chapman 2006), has provided an additional impetus to reduce feed costs by using alternate, plant-derived ingredients (Gomes et al. 1995; Hardy 1996; Sugiura et al. 1999; Carter and Hauler 2000; Kissil et al. 2000, Barrows et al. 2007a).

Replacement of fish protein and oil in the diet by plant-derived protein and oil poses technical and biological challenges. Many plant byproducts contain lower protein levels and different balances of amino acids, often with limited amounts of essential amino acids (Adelizi et al. 1998). In addition, plant meals may contain anti-nutritional factors such as trypsin inhibitors, non-digestible carbohydrates, lectins, saponins, phytates, and, possibly-allergenic storage proteins (Salunkhe et al. 1992). Replacement of fishmeal by plant meal may decrease palatability (Geurden et al 2005), which in turn could decrease consumption (Davis et al. 1995; Stickney et al. 1996). In addition, chemical composition of fillets and organoleptic characteristics of fish fed with plant diets differ from those of fish fed traditional diets (Francesco et al. 2004).

Palti et al. (2006) qualitatively compared family affiliations among the largest and the smallest fish from 20 full-sib families fed traditional fishmeal- and plant gluten-based diets to evaluate possible genotype x diet interactions in the commercial "Spring" strain of rainbow trout. The results suggested that fish that grew faster when fed a fishmeal-based diet also grew faster when fed a fishmeal-free, gluten-based diet; i.e., there was no genotype x diet interaction in this strain. Another study, conducted by Quinton et al. (2007a), found similar results for European whitefish (*Coregonus lavaretus L.*). Insignificant genotype x diet interaction was observed and there was little to no re-ranking of families between diets. Additionally, the low genetic correlation and heritability implied that selection to improve daily gain and feed efficiency on fish meal-based diets will lead to favorable responses on soybean meal-based diets. These findings suggest that current commercial strains that exhibit superior growth on fish meal-based might generally exhibit superior performance when fed plant-based diets.

This study expands the assessment of possible genotype x diet interactions in rainbow trout (*Oncorhynchus mykiss*). Several changes were made in the plant-based diet in order to test a more commercially relevant, experimental diet. Corn gluten meal, soybean meal, and a low level of wheat gluten were the primary protein sources, and the added oil was a 50% mixture of fish oil and soybean oil. I compared the growth response among 95 full-sib families fed the two diets from the widely-used Kamloop strain of rainbow trout from Troutlodge, Inc. I randomly sampled 1,000 fish from each diet group to estimate family mean and variance for size-at-age to calculate genetic correlations, and to quantitatively evaluate the possibility of a genotype x diet interaction.

Methods

Fish Stock

The Kamloop strain from Troutlodge, Inc. (Sumner, WA, USA), which has been selected for improved growth for three generations using a best linear unbiased predictor (BLUP) of breeding value, was the base population for this study.

Mating and early rearing

One-hundred-and-sixty (160) females were mated to 80 males on the basis of pedigree and breeding values using the following approach: Each male fertilized the eggs of two females, using the criterion that the parents of each cross could have no common grandparent. The parents were fin-clipped for DNA isolation and analysis. Fertilizations occurred during the week of August 9-15, 2005. Fertilization was followed by a common egg displacement procedure (Palti et al. 2006). The degree-days of all lots were matched,

and the lots reached the "eyed" stage on the same day. Family numbers then were reduced to 95 full-sib families (approximately 9,500 eggs) nested within 47 half-sib families. The same number of eggs from each family then was pooled among all families, mixed well, and randomly split into two groups that were incubated separately in hatching jars through hatching and the initiation of feeding.

The swim-up fry were placed into six 6,000-L rearing tanks supplied with spring water (12ºC). Feeding was as described below. Bi-weekly sampling of weight was continued through grow-out. Fish were transferred from rearing tanks to standard concrete grow-out raceways at an average weight of 25 g until harvest at approximately 600 grams.

Diet formulation, and feeding regime

Two diets were formulated to be isonitrogenous and isolipidic (Table 3.1). The diets were manufactured at the Feed and Nutrition Laboratory of the U.S. Fish and Wildlife Service Fish Technology Center in Bozeman, MT for 0.5 to 3.0 mm pellet sizes. The 4.5 mm pellets were produced by a commercial mill (Nelson and Sons, Murray, UT). Krill meal (5%) was added to the plant-based diet in order to increase palatability. Astaxanthin, a color-enhancing additive, was included in the 4.5 mm pellets.

Swim-up fry were fed manually at half-hour intervals through a 10-hour day. At an average weight of 25 g, feeding was shifted to semi-automated feeders delivering pellets from a conveyor into the tanks at programmed intervals. Feed amounts were calculated using a TroutLodge proprietary feeding program and adjusted weekly based on biomass estimates. Feeding rates approximated satiation feeding. During the few days

following the adjustment of feed amount, excess feed would remain in the feeders, while in the few days prior to the next weekly adjustment, all feed was consumed. Mortalities were collected daily.

Statistical analysis

Correlation between length and weight was estimated using JMP 5.0 (SAS Institute, Inc., Cary, NC) to determine whether body weight represented overall growth of the fish. Three regression analyses were produced: one for each of the diets, and one for the entire population. A paired *t*-test was used to compare growth by diet within family.

To evaluate phenotypic differences between fish from the two treatments, I used SAS Proc Mixed, (SAS Institute Inc., Cary, NC), and the following equation was used to partition variance as:

$$Y_{ijklm} = \mu + Diet_i + Rway(Diet)_{i(j)} + Sire_k + Dam(Sire)_{l(k)} + Dam(sire*diet)_{l(kj)} + \varepsilon_{i(j)klm}$$

where: Y_{ijklm} is fish size, μ is the grand mean; $Diet_i$ is the variation due to the fixed effect of diet differences; $Rway(Diet)_{i(j)}$ is the fixed effect of raceway within diet; $Sire_k$ is the effect of the k^{th} sire, $Dam(Sire)_{l(k)}$ represents the effect of the l^{th} dam within the k^{th} sire, $Dam(sire*diet)_{l(kj)}$ represents the interaction between diet and the l^{th} dam within the k^{th} sire, and $\varepsilon_{i(j)klm}$ is the random error represented by the individuals within a full-sib family for each diet-within-raceway combination. Significance values of random effects then were determined by the size of the α value *P* using the Wald *Z* statistic for covariance

parameters (SAS Institute, Inc., 1999). If $P \leq 0.05$, differences were considered significant unless noted otherwise.

In addition, Multi-Trait, Derivative-Free Restricted Maximum Likelihood (MTDFREML), a genetic analysis program developed by Boldman et al. (1991), was used to estimate (co)variance components. In this analysis, growth on each diet was considered a separate trait. The genetic correlation between growth on the fish meal-based diet and growth on the plant meal-based diet was evaluated using a missing value technique (S.D. Kachman, University of Nebraska, and L.D. Van Vleck, USDA-ARS, personal communications) with an animal model, where the error correlation was set equal to zero because each animal was tested on only one diet. The phenotypic correlation of growth on the fishmeal and plant meal diets was evaluated as a Spearman rank correlation. The non-parametric Spearman correlation was chosen because of differences in the numbers of individuals representing each family (Altman 1991). To evaluate the effect of differences in numbers of individuals sampled from each family/diet, data from full-sib families with fewer than eight individuals per diet were excluded and the correlation was re-evaluated.

Results

Overall mortality rate

Overall mortality on the plant-based diet was 4%, and on the fishmeal diet 7%. In the raceways used during the grow-out phase, it was 1% on the plant diet and 3% on the fishmeal diet. No tank or raceway effects were observed.

Weight x length correlation

Weight and length were highly correlated; hence, I used body weight as the metric of overall growth in the genotype x diet analyses. An r^2 value of 0.88 ($P < 0.0001$) was shown for the plant diet, an r^2 of 0.80 ($P < 0.0001$) was calculated for the fishmeal diet, and a combined r^2 of 0.85 ($P < 0.0001$) for both diets.

Growth

The trout fed the fishmeal diet (mean = 645.5, $\pm$ SD = 138.6 g) were significantly heavier than fish fed the plant meal diet (589.8 $\pm$ 131.1 g) ($P < 0.05$). Fixed effects analysis showed significant tank effects (Figure 3.1). A significant family x diet [$Dam(sire*diet)_{l(kj)}$] effect of 5% was detected by mixed model analysis (Table 3.2). The heritability of growth estimated by the MTDFREML animal model was 0.31 ± 0.07 on the plant diet and 0.32 ± 0.07 on the fishmeal diet, with a genetic correlation of 0.73 ± 0.13 for growth on the two diets. An additional test of phenotypic correlation between growth rate on fishmeal and plant meal diets was conducted to enable comparison of results with those of Palti et al. (2006). Although the phenotypic correlation proved significant (0.28 ± 0.10), it was much weaker than the genetic correlation (0.73 ± 0.13). When data from families with fewer than eight sampled individuals on each diet were excluded, a total of 46 families nested within 29 sires were evaluated, which led to a phenotypic correlation of 0.55 ± 013.

Discussion

Growth differences

As expected on the basis of earlier work (Barrows et al. 2007a), a significant difference in growth rate (approximately 8%) was observed between fish fed the fishmeal diet and those fed the plant-based feed. However, it may possible to match the growth on fishmeal diet with a plant protein-based diet by using more expensive protein concentrates, by avoiding soybean meal as a partial protein source, and by partial replacement of fish oil by soybean oil (Palti et al. 2006). Trypsin inhibitors, non-digestible carbohydrates, lectins, saponins, phytates, and possibly-allergenic storage proteins have been discovered in soybean meal, all of which can hinder digestion and nutrient utilization in rainbow trout (Salunkhe et al. 1992). The plant-based diet in this trial, however, contained 19.0% soybean meal, which is below the threshold atwhich performance declines. In addition, feed was processed using extrusion conditions shown to optimize performance of diets with high levels of soybean meal (Barrows et al. 2007b). Reduced feed consumption due to a preference of trout for fish meal and oil (Geurden et al. 2005) and a possible imbalance in available amino acids could have caused the reduction in growth rate.

Genotype x diet interaction

Quantitative analyses suggested a significant genotype x diet interaction, meaning that the families that grew faster on fishmeal-based diets were not necessarily the same families that grew faster on the plant-based diet. These results differ from those of Palti et al. (2006), where family x diet interaction was not observed. The different findings may be explained by the addition of soybean oil and protein to the new diet or by the different

rainbow trout strain used. The experimental design of the Palti et al. (2006) study did not allow for quantified evaluation of genetic correlation and interaction. In a similar study, Quinton et al. (2007b) observed little to no family x diet interaction in European whitefish when selecting for reduced lipid for improved feed efficiency. Little interaction suggests that current fish meal selection programs should improve future breeding selections on soybean meal diets. However, quantitative analysis of genetic relationships showed significant heritability and genetic correlation between diets, inferring the potential to select between diets for breeding.

Additional studies to characterize body composition, nutrient utilization, and energy storage sites when using alternate diet formulations have been conducted in rainbow trout to determine line x diet interaction and possible genetic x nutritional relationships (Kause et al. 2007a; Kause et al. 2007b; Quillet et al. 2007). These studies also show little heritability and low to no genetic correlation between feed efficiency and weight gain (Kause et al. 2007a), as well as little to no genetic correlation in lipid deposition and feed formulation (Kause et al. 2007b). However, Quillet et al. (2007), studying weight corrected muscle fat content, discovered that the ability of fish to store fat may differ between lines, with a higher fat line depositing more lipid content in the visceral mass, where the low line exhibited uniform distribution. With the observation of a slight line x diet interaction, indication of the ability to use combined genetic and nutritional tools to select for optimum growth production in differing rainbow trout fat strains exists.

The phenotypic correlation (0.28 ± 0.10) between the family growth on fishmeal and plant gluten diets observed here was much weaker than the correlation observed by

Palti et al. (2006). The difference can be explained partially by the genotype x diet interaction observed in this study, but the phenotypic correlation in this study was also considerably lower than the genetic correlation. Exclusion of families with fewer than eight individuals in either diet reduced the number of families in the analysis by 50%. The subsequent increase in the magnitude of the correlation from 0.28 to 0.55 showed the phenotypic correlation to be sensitive to the number of individuals sampled per family. The genetic correlation, however, was less sensitive to sample size per family. Genetic correlation was calculated using the MTDFREML animal model, which accounted for a larger sample size per family, as it incorporated data from both full-sib and half-sib relatives into the family value. Indeed, Lynch and Walsh (1998) noted that genetic correlation is often greater then phenotypic correlation.

Future implications

With the observation of high heritability and significant genotype x diet interaction, selection within and between families on the basis of phenotype is likely to prove a successful breeding strategy. That is, increased utilization of plant-based feeds might be raised via family selection in a breeding program for this rainbow trout strain. Moderate to high heritability (0.12-0.28) suggests that favorable plant-based feed utilization is a heritable trait. Moreover, moderate to high genetic correlations across diets (0.37-0.82) suggest that within- and between-family selection can improve growth performance on a plant-based diet. Classical breeding methods of nucleus broodstock selection, alternate generation selection, and walk-back selection can be implemented for

selecting those individuals displaying the highest growth within each family for optimum results (see following chapter).

Further studies characterizing markers for genes of interest could help develop a marker-assisted selection plan (Korol et al. 2007). Identifying performance-increasing alleles in individuals capable of high plant protein utilization could lead to selection of stocks with increased feed conversion with associated lower feed costs. Microarrays could be utilized to detect genes whose expression underlies increased plant utilization to identify candidate genes for QTL analysis. Gene-based methodologies may increase the potential for successful breeding programs.

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Table 3.1 Composition of the two experimental diets.

Plant Diet		Fishmeal Diet	
Ingredient	g/100g	Ingredient	g/100g
Krill meal	5.00	Fish meal	63.14
Wheat gluten	7.04	Wheat flour	23.96
Corn gluten	34.57	Fish oil	10.10
Soybean meal	18.96	Lecithin	2.00
Wheat flour	14.43	Vitamin Premix #30[a]	0.50
Fish oil	5.00	Trace min #3[b]	0.10
Soybean oil	8.43	Stay-C	0.20
Lysine-HCl	1.47		
Methionine	0.45		
Taurine	0.50		
Dicalcium phosphate	2.55		
Vitamin Premix #30[a]	0.80		
Choline Cl	0.50		
Trace min #3[b]	0.10		
Stay-C	0.20		
TOTAL	100.0	TOTAL	100.0

[a] Contributed per kg of diet: vitamin A, 10000 IU; vitamin D3, 720 IU; vitamin E, 530 IU; vitamin B12, 30ug; calcium pantothenate, 160 mg; riboflavin, 80 mg; thiamin mononitrate, 50 mg; pyridoxine hydrochloride, 45 mg; folacin, 13 mg; menadione sodium bisulfate 25 mg; biotin, 1 mg; niacin, 330 mg.

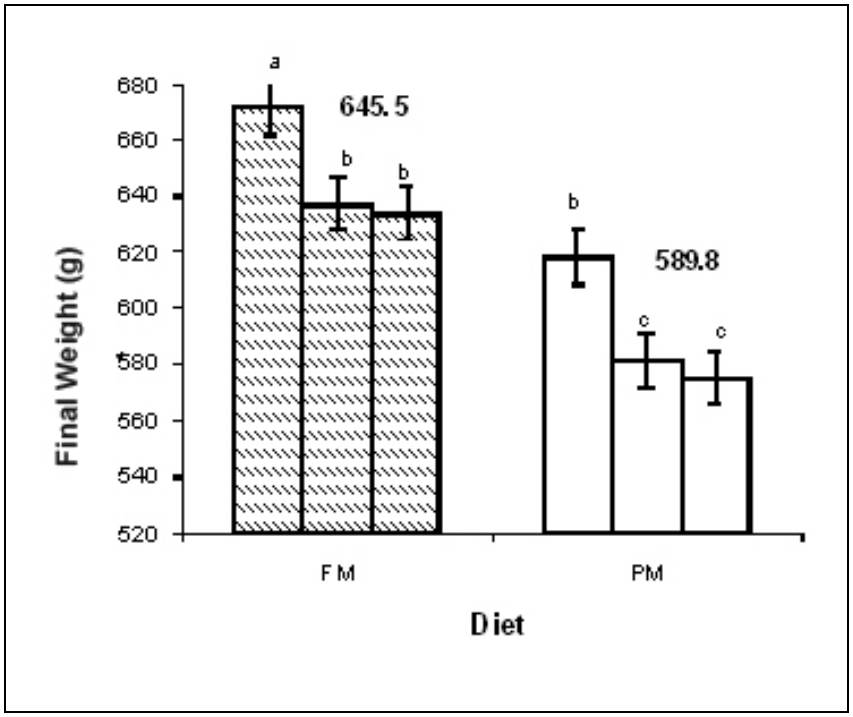
[b] Contributed in mg/kg diet: zinc, 100; manganese, 70; iron, 3; copper, 2; iodine, 1.

[C] Plant diet: 47.78% crude protein, 19.18% lipid, 3802 kcal/kg metabolizable energy. Fishmeal diet: 47.80 % crude protein, 19.07% lipid, 3861 kcal/kg metabolizable energy.

Table 3.2 Evaluation of random effects on body weight by mixed model analysis.

Source	Variance Component	Error	% Variance	*P* value
Sire	675	500	4	0.089
Dam(Sire)	1103	565	6	0.018
Dam(Sire) x Diet	835	386	5	0.015
Residual	14977	526	85	<0.001

Figure 3.1 Diet and tank effects on final body weight (g). Histogram bars indicate means of separate tanks. Final weights for the fish meal diet (FM) are represented by dashed bars and for the plant diet (PM) by white bars. Standard errors are indicated. Different letters indicate significant differences of means between tanks and diets. Mean average weights are indicated above each set of histograms for the respective diets.



Chapter IV

Future Implications

Introduction

The application of breeding programs in aquaculture dates back to 475 B.C. with carp rearing in China. In contrast to the historically long development of non-science-based breeding, the application of quantitative genetic principles to fish breeding has been limited until recently. Due to complex reproductive cycles, broodstock development and management plans can prove difficult to implement. However, popular species such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) have been kept in captivity for breeding for over 7 generations in Norway and have shown signs of tameness and domestication compared to the wild type (Gjedrem 2005). In addition, improved flesh quality can increase value in marketable products. The ability to employ selection can improve targeted traits in an aquaculture program.

Most selective breeding programs are based on individual selection. Such programs depend on a reasonably high level of heritability (>0.30) for a targeted trait (Tave 1993). More complex breeding programs might be appropriate when heritability is in the range of 0.15-0.30. Some traits, such as the ability to utilize plant-based feeds, may be measured best in common-garden experiments. In such cases genetic marker-based parentage assignment may be needed. One such selection strategy would be “walk-back’ selection. This approach exploits the high fecundity of aquaculture organisms to achieve intense selection while minimizing inbreeding. Superior individuals are selected from

genetic marker-identified families that are grown together from birth without physical tagging and without interfering with commercial operations (Doyle and Herbinger 1995). In this selection strategy, the largest individual is genotyped and placed aside as a primary breeder. Next, the second largest individual is genotyped and used as a breeder if it is from a different family than the first; if not, it is discarded. This procedure continues until a sufficient number of breeders are obtained (Doyle and Herbinger 1995). Crosses are made only between families, thereby minimizing inbreeding, but maintaining superior growth. This protocol has been implemented in rainbow trout culture and has proved to be successful (Herbinger et al. 1995).

Also appropriate for breeding for plant-based diet utilization, nucleus broodstock selection (Myers et al. 2001) selects on both genotypic and phenotypic traits, allowing the breeder to select the fittest individuals across production stocks and breed them back into the original broodstock, while also selecting other individuals for market (Figure 4.1). As with any selection strategy, advantages and disadvantages are present. Although allowing selection of breeders with optimum genotypic and phenotypic traits, inbreeding could cause problems if repeated for numerous generations. Fitness traits such as growth rate, survival, and body conformation may be reduced as inbreeding increases (Kincaid 1976). Understanding the complexity of this selection program could allow aquaculture producers to take advantage of increased growth while selecting for minimal inbreeding.

Gene Characterization

The mapping of quantitative trait loci (QTLs) is the first step toward the identification of genes and causal polymorphisms for traits of importance in agriculture,

aquaculture, and medicine (Poompuang and Hallerman 1997). Analysis of QTLs allows simultaneous estimation of both the additive gene effect and the location on the genetic map of loci influencing a trait. Commonly-researched QTLs in rainbow trout include disease resistance, growth, and spawning (Korol et al. 2007; Barroso et al. 2008). This species has a number of advantages as a research organism since numerous genetic and phenotypic distinct populations exist (Taylor 1991; Hershberger 1992). Additionally, more QTL analyses have been conducted on this fish than any other on such a wide variety of traits (Barroso et al. 2008). By practicing marker-assisted selection, i.e., by selection for markers linked to optimum performance, a breeder can select only for the traits contributing to efficient utilization of plant protein sources. Although molecular methods are time-consuming and costly, future breeding programs could perhaps benefit by understanding which genes affect increased utilization and by direct selection on the polymorphism affecting the trait of interest.

Microarray studies also have the ability to identify genes underlying expression of a trait. Microarray technology utilizes nucleic acid hybridization techniques and advanced computational data analysis methods to evaluate mRNA expression profiles of thousands of genes within a single assay (Sha 2006). Using microarrays to monitor expression of thousands of proteins simultaneously, geneticists can study how these genes function and follow their expression under different conditions (Amaratunga et al. 2004). This technique allows identification of important genes underlying aquaculture performance (i.e., growth). Subsequent QTL analysis of candidate genes could identify what factors influence the trait, thereby allowing producers to select for individuals expressing performance-increasing variants. Once the initial characterizations of plant

meal-utilization genes are complete, broodstock animals then could be fin-clipped and used for DNA extraction to determine genotype. After genotypes are identified, breeders could apply the information to family members, selecting animals for optimum performance on plant-based protein diets.

From this research I was able to contribute:

- Comparison of two marker based parental allocation systems with associated advantages and disadvantages
- Nutrition information on plant-based diets for aquaculturists who produce rainbow trout
- Genetic evidence of genotype x diet interaction for a widely used commercial rainbow trout strain which would prove useful in breeding

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Figure 4.1 Representation of nucleus broodstock selection program.